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Embryological exposure to valproic acid disrupts morphology of the deep cerebellar nuclei in a sexually dimorphic way

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ABSTRACT

Autism spectrum disorders (ASD) is diagnosed in males at a much higher rate than females. For this reason, the majority of autism research has used male subjects exclusively. However; more recent studies using genetic sex as a factor find that the development of the male and female brain is differentially affected by ASD. That is, the natural sex-specific differences that exist between male and female brains lead to sexually dimorphic expressions of autism. Here we investigate the putative sexual dimorphism that exists in the deep cerebellar nuclei of male and female rats exposed to valproic acid (VPA) on embryological day 12.5. We find natural sex-specific differences in adult nucleus area, length, and estimated cell populations. Therefore VPA exposure during embryology creates some sex-specific deficits such as higher cell counts in the VPA males and lower cell counts in the VPA females. At the same time, some effects of VPA exposure occur regardless of sex. That is, smaller nucleus area and length lead to truncated nuclei in both VPA males and females. These deficits are more pronounced in the VPA males suggesting that genetic sex could play a role in teratogenic susceptibility to VPA. Taken together our results suggests that VPA exposure induces sexually dimorphic aberrations in morphological development along a mediolateral gradient at a discrete region of the hindbrain approximate to rhombomere (R) 1 and 2. Sex-specific disruption of the local and long-range projections emanating from this locus of susceptibility could offer a parsimonious explanation for the brain-wide neuroanatomical variance reported in males and females with ASD.

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1. Introduction

Autism belongs to a spectrum of neurodevelopmental disorders (ASD) characterized by specific behavioral abnormalities (American Psychiatric Association, 1994). A pervasive trend of this disorder is that males are more likely to be diagnosed with ASD than females (Lord et al., 1982; Fombonne, 1999, 2003). This male biased ratio has recently been connected to specific genetic differences between men and women (Lepage et al., 2014; Verma et al., 2013). In fact, some researchers have theorized that autism is related to abnormal brain growth that over-develops "male" cortical regions (Baron-Cohen, 2002). This theory posits that sexually dimorphic neuro-anatomical differences lead to sex-specific behavioral phenotypes of autism (Baron-Cohen et al., 2003, 2005; Craig et al., 2007). To that end, some recent studies have suggested that there are significant sex-specific differences between men and women diagnosed with ASD (Beacher et al., 2012; Chou et al., 2011; Gillies et al., 2014; Lombardo et al., 2012).

The human connection between fetal exposure to the antiepileptic compound VPA and the expression of autism was well established in the early 1990s (Christianson et al., 1994). Following this etiological link, the Valproic acid (VPA) animal model of autism was developed (Rodier et al., 1996). This rodent model shares striking behavioral and anatomical features with human ASD (for review see Markram et al., 2007). Furthermore, there is evidence that male and female rats exposed to VPA have fundamental behavioral and anatomical differences that significantly differ from their sex-matched controls (Kim et al., 2013; Schneider et al., 2008; Mychasiuk et al., 2012; Kataoka et al., 2013; Hara et al., 2012). Therefore we were interested in using this animal model to continue investigating the nature of sexually dimorphic expressions of autism.

The cerebellum and anterior hindbrain is one of the regions that is highly susceptible to the teratogenic effects of embryological exposure to VPA (Arndt et al., 2005). Therein, it was demonstrated

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that animals exposed to VPA had aberrant anatomical features in areas such as the vermis and DCN. These abnormalities are linked to disruptions of *hox* genes (Stodgell et al., 2006) and cell proliferation genes further upstream, such as PTEN (Zhang et al., 2003). The expressions of both of these factors are synergistically altered by the sex of the organism (Christiansen et al., 2002; Wang et al., 2011). Therefore the focus of the current study was on this area of the hindbrain/cerebellum.

Unbiased stereological analysis revealed natural dimorphisms in the morphology of the fastigial nucleus, interpositus nucleus, and dentate nucleus of these animals. Males generally had larger longer nuclei with higher total estimated cell counts. VPA exposure further altered these differences in a sex-specific manner. These anatomical aberrations were distributed both mediolaterally and rostrocaudally along the poles of the nuclei. Therein anatomical changes were more prevalent in the males than the females along both gradients. Furthermore the changes themselves were dimorphic between male and female VPA animals, with males having higher cell counts and females having lower cell counts than their sex-matched controls. Taken together these results demonstrate a sexually dimorphic effect of embryological VPA exposure on the development of the DCN.

2. Methods

2.1. Subjects

Thirty Long Evans Hooded rats from 15 litters were used in this study (VPA – 5 male, 10 female; Saline – 5 male, 10 female). All were offspring from timed-pregnant females that were shipped to the University of Delaware animal facility on gestational day (GD) 4 or 5 from Harlan Laboratories (Frederick, MD). VPA and Saline control rats were obtained. During the first postnatal week litters were culled to eight pups (4 males and 4 females). On PND21, pups were weaned from their mothers and housed in groups of same-sex littermates in cages the same size that they were reared in, until the start of the experiment. One male and one female from each litter was selected for use from the laboratory of Dr. Mark Stanton and shipped to Indiana University ~ postnatal day (PND) 50 from the University of Delaware. For five litters only 1 female was selected as males were being used for another experiment. A complete method detailing the procedures for VPA dosing and route of administration has been previously reported (Stanton et al., 2007a,b).

2.2. Histology

At PND 90 adult rats were euthanized by urethane overdose via intraperitoneal injection (.25 mg/100 g). Animals were then perfused transcardially with cold 0.9% saline solution followed by 100 ml of 4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Volume of perfusates was closely monitored to standardize fixation parameters between animals. Brains were cryoprotected overnight in 30.0% sucrose and 0.1 M phosphate buffer, pH 7.4. Brain tissue was mounted frozen and sliced coronally (40 μm) through the entire cerebellum using a freezing microtome. The tissue sections were float-mounted in phosphate-buffered-saline (PBS), dehydrated with ascending ethanols and thionin stained. Tissue histology procedures were strictly timed and monitored between animals to standardize general shrinkage artifacts imposed by the staining process. All data quantification was carried out at 4× (area, length) or 40× (cell counting) under brightfield illumination using a microscope (Nikon Eclipse 80i; Nikon Instruments; Melville, NY, USA) and the Stereo Investigator software (MBF Bioscience; Williston, VT, USA). This software has a number of histological probes and allows full investigation along the *x*, *y*, and *z* planes of a tissue section (see Fig. 1).

2.3. Unbiased stereological quantification

Unbiased stereological quantification was carried out under brightfield illumination using a microscope (Nikon Eclipse 80i; Nikon Instruments; Melville, NY, USA) and the Stereo Investigator software (MBF Bioscience; Williston, VT, USA). Coronal sections were cut through the DCN at 50 μm, and every other section was kept for thionin staining. For each animal the left or right DCN was randomly selected for analysis. All analyses were carried out blindly. The anatomical lengths were quantified along the rostrocaudal axis for the interpositus nucleus (IPN), dentate nucleus (DN), and fastigial nucleus (FN) by determining the number of tissue sections for which each nucleus was visible. This produced a number of sections per animal. On each tissue section used in the length analysis the contours were drawn around the region of interest generating values that approximated the two dimensional area in mm^2. The sections containing the three largest area measurements were then selected for unbiased stereological cell count estimates.

The optical fractionator probe was then used to estimate the cell number contained within each region of interest. These numbers were divided by the area and

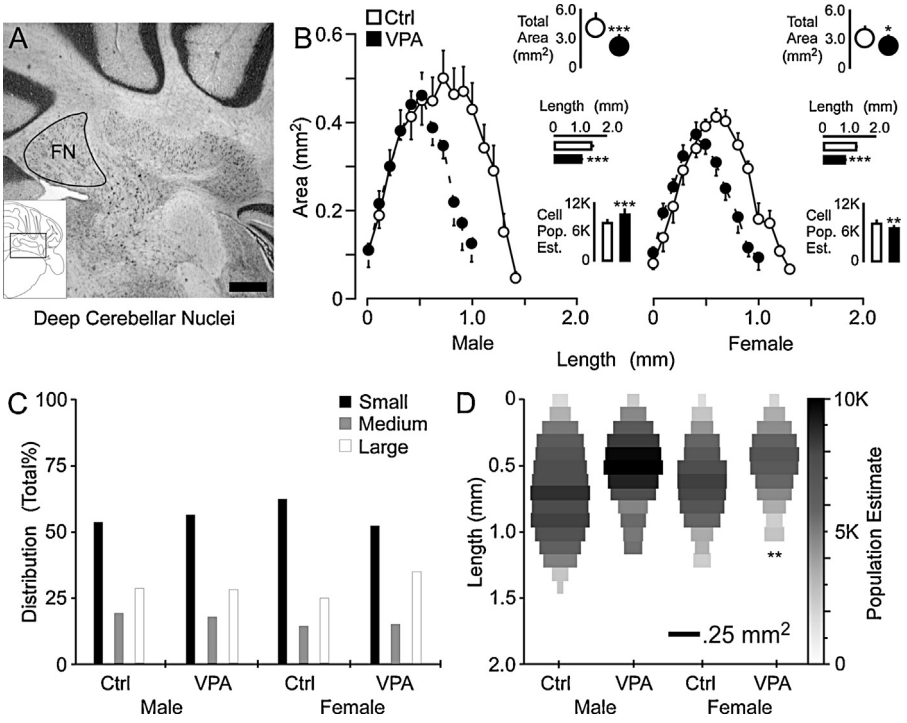

Fig. 1. Neuroanatomical changes to fastigial nucleus of male and female rats induced by embryological exposure to VPA. (A) Coronal section showing fastigial nucleus (contour) of the cerebellum (inset diagram). (B) Lineplots comparing the VPA induced differences in nucleus area throughout the length of the fastigial nucleus between age and sex matched controls. Insets plots show the quantitative difference between VPA animals and sex-matched controls for FN area (top), length, (middle) and cell count (bottom). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. (C) Relative distribution of cell diameters in the fastigial nucleus of sex-matched VPA and control animals. (D) Schematic showing a horizontal representation of the fastigial nucleus for sex-matched VPA and control animals. $^{**}p < .01$.